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Studying the Nuclear Import of Thyroid Hormone Receptor Alpha using a Mammalian Two-Hybrid System

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by

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Abstract

Thyroid Hormone Receptor α1 (TRα) is a nuclear hormone receptor that plays a vital role in differentiation, development, and maintenance of homeostasis in mammals. TRα functions as a transcriptional repressor when thyroid hormone (TH) is not bound, but upon TH binding TRα undergoes a conformational change which causes TRα to function as a transcriptional activator. TRα shuttles rapidly between the nucleus and the cytoplasm across the nuclear membrane. This thesis research focused on the mechanisms regulating nuclear import of TRα. Prior studies have shown that TRα contains two nuclear localization signals (NLSs) and that TRα can be imported into the nucleus *in vitro* by the classical nuclear import pathway mediated by importin α and importin β.

Here, to determine the specific mechanism of nuclear import and the specific importins that mediate this import in mammalian cells, both *in vitro* and *in vivo* binding methods were considered and were optimized for use. First, in order to perform *in vitro* binding experiments, optimization of protein expression of TRα and importin αs was performed with limited success as expression in *E. coli* proved extremely difficult even under a vast array of conditions. After it was clear that overexpression in *E coli.* was not possible with current expression vectors, a different method to study nuclear import of TRα was developed.

A mammalian two-hybrid system was developed and optimized for use in both HeLa (human) and NIH3T3 (mouse) cells using positive controls. Genes encoding importin α isoforms were subcloned into the pACT vector and confirmed by sequencing while genes encoding TRα domains: A/B, DBD, Hinge, and LBD were unable to be successfully cloned into the pBIND vector due to time constraints as rapid screening was unable to be performed due to the small size of these inserts. Taken together this thesis research provides a powerful method to study protein-protein interactions among transcription factors and importins, which can be used to understand the mechanisms regulating both the nuclear import and export of TRα.

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Acknowledgements

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General Introduction

Transcription factors, sequence-specific DNA binding factors, are proteins that regulate the transcription of genes either positively or negatively by promoting or blocking the recruitment of RNA polymerase to specific genes (Latchman, 1997). Transcription factors are essential for both tissue specific-gene expression and regulation of gene activity in response to specific stimuli. One superfamily of transcription factors is the nuclear receptor superfamily, which describes a diverse assortment of transcription factors including both nuclear hormone receptors and orphan nuclear receptors. Nuclear hormone receptors (NHRs) are receptors for which hormonal ligands have been identified, whereas orphan receptors have no known ligand. Lipophilic hormones act as ligands for these receptors and are able to traverse the plasma membrane into the cell interior in order to bind to NHRs. All nuclear receptors have a common modular structure generally consisting of a N-terminal regulatory domain (A/B), a DNA-binding domain (DBD), a Hinge region, a Ligand binding domain (LBD), and a variable C-terminal domain.

One nuclear hormone receptor of the type II superfamily, the thyroid hormone receptor (TR), plays a vital role in differentiation, normal development, and maintenance of homeostasis in many organisms. This type II nuclear receptor superfamily includes the vitamin D receptor and the retinoid X receptor, as well as steroid hormone receptors such as estrogen, androgen, and glucocorticoid receptors (Yen, 2001). TRs bind thyroid hormone (TH) as well as specific DNA-sequences, thyroid hormone response elements (TREs), which are in enhancer elements located in promoter regions. TRs like other nuclear receptors contain an A/B domain that is essential for transactivation, a central

DBD consisting of two zinc finger motifs, a Hinge region that contains a conserved nuclear localization sequence (NLS), and a carboxy-terminal LBD that binds TH. In most instances, TRs function as transcriptional repressors when TH is not bound and as activators upon ligand binding (Zhang & Lazar, 2000). TR has been shown to shuttle rapidly between the nucleus and the cytoplasm and since TR has a size of ~46 kDa passive diffusion into and out of the nucleus is not possible in mammalian cells. TR seems to also have a co-existing passive route in amphibian oocytes (Bunn et al., 2001). The import and export of TRα1 (hereafter referred to as TRα for simplicity) have been studied and it has been shown that there are two NLSs located within the TRα protein; both the A/B domain and Hinge domain of TRα have NLSs that are sufficient for nuclear import (Mavinakere and Allison, manuscript in prep). Data have shown that TRα can be imported into the nucleus *in vitro* by the classical nuclear import pathway mediated by importin α and importin β (Roggero, 2008). The goal of this thesis research was to better understand the molecular mechanism of TRα nuclear import by developing a suitable method to study TRα-importin α interactions. The following sections provide background on both TR and the classical nuclear import pathway.

Thyroid Hormones

Thyroid hormones are essential for regulating development, growth, and metabolism of many vertebrates. These hormones act on almost every cell in the body and are responsible for such actions as regulating the basal metabolic rate, neuronal maturation, bone growth, and heat generation in humans. The major hormone secreted from the thyroid gland is 3,5,3',5'-tetraiodothyronine (thyroxine or T4) and to a lesser extent 3,5,3',triiodothyronine (triiodothyronine or T3) (Figure 1). THs are tightly

regulated through a complex endocrine pathway that begins in the hypothalamus located in the brain. The hypothalamus releases thyrotropin releasing hormone (TRH), which subsequently travels through the bloodstream of the hypo-hypophyseal portal system. TRH production is controlled through the regulation of enzymes such as PC-1, PC-2, and carboxypeptidase E (Werner et al., 2005). TRH stimulates the release of thyroid stimulating hormone (TSH) from the pituitary gland. TSH then stimulates the thyroid gland to secrete T4. T3 is secreted to a lesser extent than T4 because free T3 in circulation causes autoinhibition through a negative feedback loop in which free T3 down regulates the production of TRH which in turn down regulates TSH (Berne and Levy, 1990). T4 is the prohormone and is converted by 5' deiodination of the outer ring to the more biologically active T3 in peripheral tissues. This deiodination is facilitated by both type I and II deiodinases depending on the location as these deiodinases are tissue specific. T3 binds to its receptors with approximately 10 to 15 fold higher affinity than T4. The saturation levels for nuclear receptors with TH are approximately 75% in the brain and pituitary and 50% in the liver and kidney. TH exerts its major effects at the genomic level through binding to TR, which is the receptor that mediates most actions of T3 (Baumann, 2001).

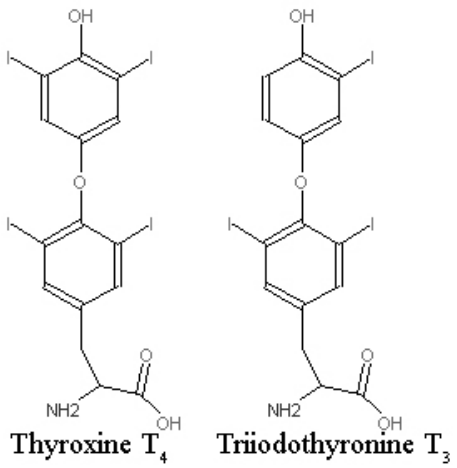

Figure 1- Thyroid Hormones

Chemical structure of thyroxine and triiodothyronine drawn in Java Molecular Editor

Modular Structure of Thyroid Hormone Receptor α1

The AB domain, amino acids 1-52 contains a constitutive N-terminal activation function (AF-1) (Figure 2). A novel monopartite NLS has been identified in our lab in this region and studies have shown that the AB domain itself confers a nuclear distribution on a cytosolic protein (M.S. Mavinakere and L.A. Allison, unpublished results; Powers, 2009).

The centrally placed DNA binding domain (DBD), amino acids 52 to 126 is the most conserved region among all NRs. The DBD consists of a 66-residue core made up of two cysteine-rich zinc-finger binding motifs that bind specifically to TREs. These zinc fingers are small structural motifs that coordinate one or more zinc ions to help stabilize their folds. The DBD also contains two α helices, a COOH extension, and several sequence elements referred to as P,D,T, and A boxes. These sequence elements are what makes each DBD on different NRs specific for certain DNA element targets (Germain, 2006). The DBD functions predominantly in DNA binding, but also participates in TR-

RXR heterodimerization (Bain et al., 2007) and acts as a target for post translational modification (Germain et al., 2006).

The Hinge domain, amino acids 126-222, connects the DBD with the LBD and forms a turn in the structure of the protein. Previous subcellular localization research shows that the Hinge region of TR α contains a NLS that targets the protein to the nucleus and also aids in DNA-binding and hormone binding activities (Lee and Mahvadi, 1993; Powers, 2009).

The ligand binding domain (LBD) consists of amino acids 223-490. The LBD is responsible for hormone binding, homo/hetero dimerization, molecular interactions with heat-shock-proteins, and transcriptional activation and repression. All of these functions are regulated by ligand-dependent conformational changes. The ligand binding pocket can be divided into three regions with region I being a largely hydrophobic region containing only a single polar residue which forms a hydrogen bond with a hydroxyl group of THs (Figure 1). This hydroxyl group has been shown to be important to function, as TH analogues that lack a phenol cannot modulate normal TH function. Region II is composed mainly of nonpolar residues that interact with the phenolic rings of THs, whereas region III is composed mostly of polar residues and interacts with the oxyacetic acid functional group of THs (Bleicher, 2008). The LBD has also been shown to contain several novel NESs that follow both CRM1-dependent and CRM1-independent nuclear export pathways (M.S. Mavinakere and L.A. Allison, unpublished results; Powers, 2009).

Figure 2- Thyroid Hormone Receptor α1

Illustration of TR showing the modular structure and distinct domains: AB aa1-52, DBD aa53-126, Hinge aa127-222, and LBD aa223-490. Also shows the identified NLSs and NES located within TR.

Regulation of TR

Transcription factors such as TR need strong regulation which can be achieved by regulation of expression, protein stability, protein activity by post-translational modifications, and subcellular localization (Yin and Meir, 2009). Changes in levels or location of functional TRs can have detrimental effects. Two TR genes are expressed in mammalian tissues, TRα and TRβ encoded on human chromosomes 17 and 3 respectively. Both isoforms bind T3 with a dissociation constant of 10^{-9} to 10^{-10} M and both mediate TH-regulated gene expression (Yen et al., 2006). Due to alternative splicing there is additional heterogeneity of TRs as the splicing of the initial RNA transcript of the TRα gene generates two mature mRNAs that each encode two proteins: TRα1 and TRα2. TRα2 is unable to bind T3 due to a 122 amino acid carboxyl terminus that replaces a region in the LBD of TRα1 that is essential for TH binding. TRα2 is able to bind TREs albeit with a much lower affinity than TRα1 but is unable to transactivate TH-responsive genes. The TR beta gene encodes three TR isoforms, TRβ1, TRβ2, and TRβ3, which

have differing amino-terminal regions. The three major isoforms TRα1, β1, and β2 bind T3 with high affinity and mediate thyroid hormone-regulated transcription. Sequence analysis indicates that there is a high degree of sequence homology in the DBD (86%) and the LBD (82%) among these major forms (Leo and Cheng, 2000). The A/B and Hinge regions on the other hand show little to no sequence similarity between TRα and β isoforms (Oppenheimer, 1996). The expression of TRs is tissue-dependent as TRα1 is expressed predominantly in the heart, bone, and brain, whereas TRβ1 is more abundant in the liver, kidney, and thyroid. TRβ2 expression is limited to the pituitary, hypothalamus, retina, and inner ear. TRβ3 on the other hand is expressed mainly in the heart and kidney. TR expression is also developmentally-regulated as certain isoforms are present only at certain points in development (Ahmed et al., 2008).

Transcriptional Regulation by TR

The transcriptional activity of the TRs is regulated at multiple levels and controlled tightly by many different protein factors. One important level of regulation of activity of TR is the diversity of TREs. Comparison of TREs identified in T3-responsive genes has shown a core consensus sequence of the hexanucleotide (A/G)GGT(C/A/G)A. Promoters of T3 genes contain pairs of these TRE half-sites. The half-site binding motif can be arranged as a divergent repeat, a direct repeat, or as an inverted repeat. There is variation in the amount of spacing between the two half-sites as the spacing is six nucleotides in the divergent repeat, four nucleotides in the direct repeat, and no spacing in the inverted repeat motif. Transcription is regulated by the ability of TR to recognize the consensus sequence of the half-site binding motifs in different arrangements. The binding affinity of TR with TRE is dependent on TR subtype, TRE motif, and T3 binding. The

type of TRE located on the promoter of the target gene affects the magnitude and sensitivity of the transcriptional response (Tsai and O'Malley, 1994; Katz and Koenig, 1993).

TR binds to TREs not only as homodimers but also as heterodimers with other members of the receptor superfamily, such as the vitamin D receptor (VDR), all subtypes of the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Heterodimerization with RXR dramatically increases the binding of TRs to TREs, the responsiveness of TR to T3 and the transcriptional activation. Heterodimerization with RAR also provides a different affinity for T3 and TREs, but more importantly the RAR/TR dimer can recruit both corepressors and coactivators, whereas RXR/TR dimers can only recruit coactivators. The VDR/TR heterodimer allows for yet another more intricate regulation of TR action by taking advantage of a polarity-dependent ligand responsiveness that is achieved by the VDR/TR heterodimer (Tsai and O'Malley, 1994; Yen, 2001).

TR regulates transcription by recruiting coactivators and corepressors to the promoter regions of target genes. TR is unique in the fact that is able to modulate gene expression both in the presence and absence of TH. TR recruits corepressors such as N-CoR which mediates ligand-independent inhibition of gene transcription. TR has been shown to interact with NCOA3 and NCOA6 leading to a strong increase of transcription of target genes when bound to TH due to chromatin remodeling and transcriptional activation (Zhang and Lazar, 2000; Bain et al., 2007).

An Overview of Nucleocytoplasmic Transport

The cytoplasmic and nuclear compartments of eukaryotic cells are separated by the nuclear envelope (NE). This separation facilitates the precise regulation of cellular processes such as gene expression, signal transduction, and cell cycle progression (Cyert, 2001). This spatial separation between the cytoplasm and the cell nucleus necessitates the continuous exchange of macromolecular cargo across the NE. The subcellular localization of TR is regulated by nucleocytoplasmic shuttling as it is unable to function as a transcriptional repressor or activator if the protein is not localized to the nucleus. The subcellular location of TR is tightly controlled through complex interactions with several proteins and studies have shown that TR has a predominantly nuclear localization (Bunn et al.,2001). The subcellular localization of TR is fundamentally controlled by both nuclear localization sequences (NLSs) and a nuclear export sequence (NES) although other protein interactions also play a role. These sequences are both essential and sufficient for passage through the nuclear pore complex (NPC).

NPCs are large multiprotein complexes that support both passive diffusion and also facilitate receptor-medicated translocation of proteins and ribonucleoprotein complexes. These NPCs allow passive diffusion of small proteins, less than 40kDa, and other small molecules such as ions, but restrict translocation of larger molecules to those with NLSs and NESs (Lange et al., 2007). The vertebrate NPC is a ~120 MDa protein complex made of about 30 different proteins, which are referred to as nucleoporins (Nups). These Nups are repetitively arranged as distinct subcomplexes that form the NPC. The NPC contains an eightfold symmetric central framework known as the spoke complex which encloses a central pore that is ~50 nm long. Enclosed by the central

framework is the central pore of the NPC, which has a diameter of ~65 nm at its cytoplasmic and nuclear periphery and ~45 in the midplane of the NPC/nuclear envelope (Beck et al. 2004, 2007). This central pore mediates the nucleocytoplasmic traffic including both small molecules and ions as well as the selective transport of macromolecular cargo with a NLS or NES and diameter up to 39 nm.

The NPC is comprised of 30 different proteins which can be grouped into three distinct classes. The first class of nucleoporins is the transmembrane group of proteins that have transmembrane α helices and a cadherin fold. These make up the outermost features of the NPC central framework and are thought to assist in anchoring the NPC to the nuclear envelope. The next class contains beta-propellers and α-solenoid folds which localize toward the inside of the NPC. The third class contains the conserved sequence motif of phenylalanine-glycine (FG)-repeats in combination with a coiled-coil fold and is thought to contribute to the formation of the NPC's inner central framework and the peripheral structures (Devos et al. 2006; Schwartz 2005; Tran and Wente 2006). FG-repeat domains are responsible for the interaction between transport receptors carrying cargo with the appropriate nuclear signal and the NPC. These FG-repeats are found in about one-third of the nucleoporins and are essential for nucleocytoplasmic trafficking. The interactions between FG-repeats and various transport receptors have been studied and structures have been solved showing the primary interaction between the phenylalanine ring of the FG-repeat core and hydrophobic residues on the surface of the receptor (Isgro and Schulten 2005, 2007). These FG-domains have been shown to have little to no secondary structure and are very disordered which allows them to be mobile

and flexible within the NPC. Studies have estimated that each NPC is comprised of at least 128 FG-domains containing ~3500 FG-repeats (Strawn et al. 2004).

Sequence specific signals, NLSs and NESs, facilitate recognition and binding by specific receptors, referred to as karyopherins, with those involved in import and export termed importins and exportins respectively. Karyopherins are a conserved family of mobile targeting receptors that mediate the bidirectional trafficking of macromolecules through the NPC across the nuclear envelope. Over 20 karyopherins have been identified in the human genome and the classification of karyopherins has proved difficult leading to a large amount of confusion regarding the naming of karyopherins. They cannot be classified solely based on their cargo collection as many cargos can be targeted by several different karyopherins. The first nucleocytoplasmic transport pathway that was studied, the classical nuclear import pathway, has established the mechanistic principles that govern all nucleocytoplasmic trafficking pathways (Lange, 2007).

Generally karyopherins bind directly to the cargo proteins although this not the case in classical nuclear import (Figure 3). Classical nuclear import is mediated by both importin α and importin beta. Importin α is essential for classical nuclear import and functions as an adaptor protein for importin beta. Importin α contains an importin beta binding domain (IBB) which binds to importin beta forming a heterodimer that serves as the classical nuclear import receptor protein. Importin α specifically recognizes and binds to cargo proteins which contain NLSs, whereas importin beta is responsible for docking to the NPC and mediating translocation through the NPC into the nucleus (Gorlich et al., 1995). Once the import complex reaches the nucleus, it is dissociated by RanGTP. Binding of RanGTP to importin beta causes a conformational change that permits the

release of the importin α cargo complex. The importin α cargo complex is then dissociated by a combination of the autoinhibitory region on the IBB domain, various Nups, and the export receptor of importin α, CAS/RanGTP. This dissociation of importin α/cargo complexes occurs at the NPC and is more energetically favorable to occur on the nucleoplasmic side of the NPC. Once the cargo is released, importin α is then exported from the nucleus into the cytoplasm by CAS/RanGTP and importin α is ready to function as an adaptor protein once again (Hood and Silver, 1998; Kutay et al.,1997).

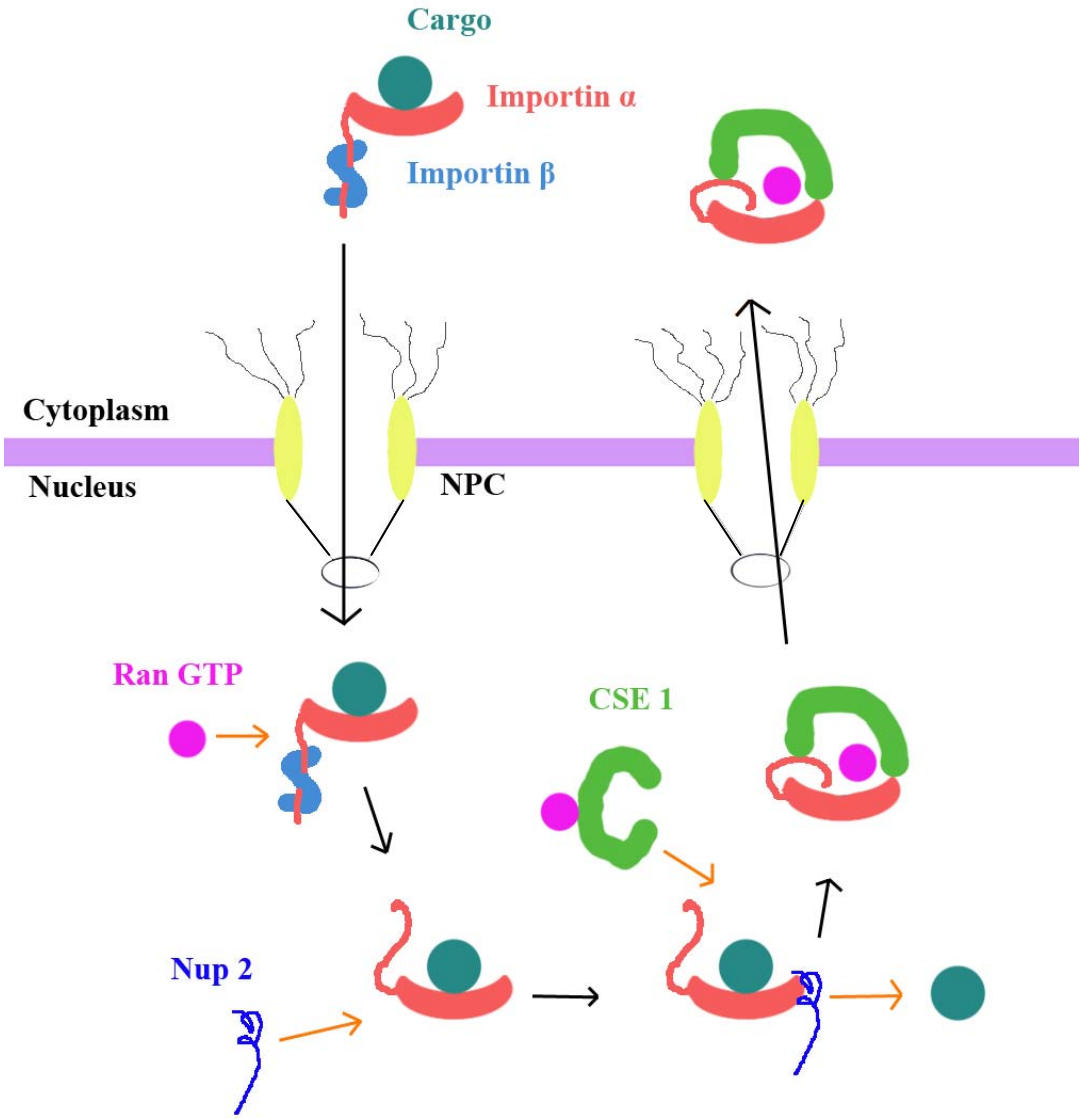

Figure 3 – The classical nuclear import cycle

The classical nuclear import cycle showing a cargo containing a cNLS that binds to the heterodimeric import receptor, import alpha/importin beta. The importin/cargo complex transverses the NPC and RanGTP binds to the complex causing a conformational change in importin beta, which releases the IBB region of importin alpha. The IBB domain, together with Nup2 and Cse1, facilitates cargo dissociation and delivery of the cargo in the nucleus. Lastly, importin alpha is recycled back to the cytoplasm by the export receptor, Cse1, in complex with RanGTP (Adapted from Figure 1 Lange, 2007).

Classical Nuclear Localization Signal

The first step in the classical nuclear import pathway is the binding of importin α to a cargo protein that contains a NLS. The best characterized transport signal is called the classical NLS (cNLS) and consists of either one (monopartite) or two (bipartite) stretches of basic amino acids. The monopartite cNLS is best characterized by the SV40 large T antigen NLS P**KKKRR**V whereas the bipartite cNLS is best characterized by the nucleoplasmin NLS **KR**PAATKKAGQA**KKKK**. Structural and thermodynamic studies have shown that a monopartite cNLS requires a lysine at the first position (P1) followed by basic residues in P2 and P4 giving a loose consensus sequence of K(K/R)X(K/R), where X is any amino acid (Conti and Kuriyan, 2000; Fontes et al,2000; Hodel et al, 2001).

This bipartite sequence contains two clusters of basic amino acids, separated by a space of about 10 amino acid residues, although studies have shown that the spacer region can be even as long as 20 amino acids. Mutagenesis experiments have shown that the first basic cluster of the bipartite NLS is essential as the second sequence alone is not sufficient to mediate nuclear import. The second inactive basic sequence can be made functional by flanking it with neutral and acidic residues such as PAA**KKKK**LD. The basic sequence is only activated for nuclear import when the PAA sequence is found upstream of the basic sequence (Makkerh et al., 1996).

It has been shown that the binding affinity of a cNLS for importin α measured *in vitro* correlates with the steady state nuclear accumulation and import rate of the corresponding cNLS cargo *in vivo* (Hodel et al., 2006). A functional cNLS has been

shown to have a binding constant of ~10nM for importin α. Computer simulations have shown that the import rate of cNLS cargo depends strongly on the rate of formation of the import complex (Riddick, 2005). Import and subsequent accumulation of cNLS cargo in the nucleus depends both on the affinity of the cNLS cargo for importin α and by the concentration of the importin α receptor itself.

Importin α/cNLS interaction

The molecular interaction between importin α and cNLS cargo has been studied using x-ray crystallography techniques (Conti and Kuriyan, 2000 ; Fontes et al., 2000; Conti et al., 1998; Fontes et al., 2003). These structural studies have shown that importin α is composed of a large curved domain consisting of ten armadillo (ARM) motifs, each of which is constructed from three α-helices, and a flexible domain IBB domain, which is required for both binding to importin beta and cargo dissociation (Figure 4). These ARM repeats consist of around 40 amino acids in tandem repeats. The IBB domain either acts *in trans* with importin beta or *in cis* with the cNLS-binding groove. The IBB domain acts as a competitive inhibitor regulating binding of cNLS cargo to the binding groove. The IBB domain contains a sequence of basic amino acids that resemble a cNLS and importin α itself was just another cargo molecule for importin beta early in the evolutionary history of nuclear transport. This regular sequence of repeated ARM motifs generates a slightly curving, elongated molecule where the major and minor cNLS-binding sites are located within a shallow groove on the concave face. Both binding pockets are formed by conserved tryptophans and a set of asparagines which are located four residues downstream. These asparagines residues form hydrogen bonds with the cNLS sequence.

Also negatively charged glutamate and aspartate amino acids interact with positively charged lysine residues found in the cNLS sequence (Yang et al., 2010).

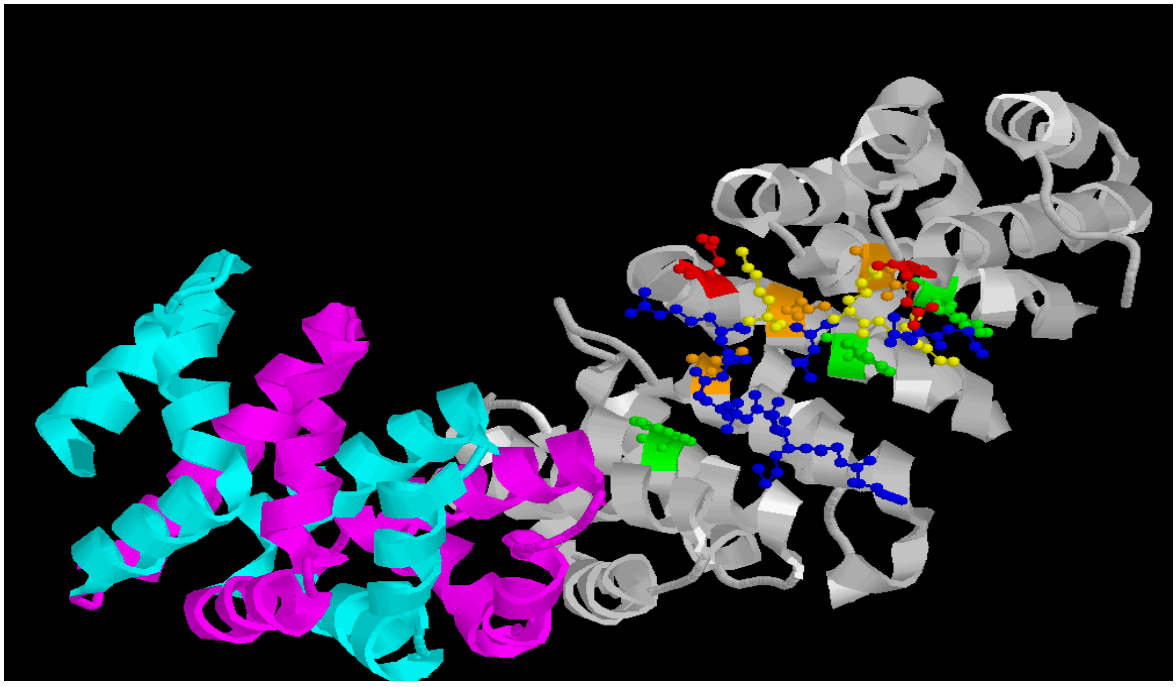

Figure 4- Crystal Structure of Karyopherin α 2 with attached ligand (NLS from Androgen Receptor) visualized with RasMol

First, the entire structure was displayed as cartoons showing the α helixes and turns. Next, the AR NLS Hinge region 611-625, the ligand, was selected, colored blue, and displayed as ball and stick. Next, specific lysine residues (K620, K622, K623) found in the NLS sequence of the Hinge domain were selected and colored yellow. Mutations in these lysine residues were shown to cause a decrease in binding to importin α and a mutation from lysine to alanine in all three residues completely abolished binding. Next, three key asparagines residues in importin α (N146, N188, N235) were selected, colored orange, and displayed as ball and stick. These asparagines residues form hydrogen bonds with the NLS sequence. Binding at the major site happens as an insertion between three tryptophan residues in importin α (W142, W184, W273), colored green and displayed as ball and stick. Specifically placed negative charged amino acids such as glutamate 107 and aspartate 192 interact with positively charged lysine residues found in the NLS sequence previously mentioned. These two negatively charged amino acids were colored red and displayed as ball and stick. Importin α contains ten tandem armadillo (ARM) repeats each repeat having three α helixes and containing about 40 amino acids. The last

four repeats 7-10 have been selected and colored to show these repeats. ARM repeat 8 and 10 were colored cyan and ARM repeats 7 and 9 were colored magenta.

Importin Alpha Isoforms

The importin α gene family belongs to a larger family of eukaryotic armadillo (ARM) domain proteins, which includes beta-catenin. The impotin α gene family is very diverse and has undergone multiple rounds of duplications and lineage-specific expansions. Most animal importin αs can be grouped into one of three conserved classes, referred to as α 1, α 2, and α 3. Even though all importin αs can mediate nuclear import by binding to importin beta, each isoforms is specialized to mediate the import of distinct repertoires of NLS-cargos. Most plant and fungal importin αs resemble animal α 1 genes and are referred to as α1 like. This suggests that α 2 and α 3 genes arose from an animal α 1 like progenitor. Importin α 1,2, and 3 genes are restricted to metazoans and occur in bilateral and nonbilateral animals. Intron organization of α 1 like genes found in plants and fungi are very similar to that of animal α 1 genes. Gene structures of importin α 2s and α 3s are more similar to one another than to α 1s mostly due to the fact that intron positions that are conserved in plant α 1 genes are mostly absent in animal α 2s and α 3s (Mason et al., 2009).

Currently six different importin α isoforms have been identified: importin α 1,3,4,5,6, and 7. Based on sequence analysis human importin αs can be grouped into three subfamilies. Within a subfamily the identity is at least 80% with some subfamily members being even closer in similarity, whereas members of different subfamilies have around 50% sequence identity (Lange, 2007). Some tissue specificity in the expression of these isoforms has been shown although most isoforms are expressed in all tissues with

varying amounts. It has also been shown that some proteins with NLSs bind to a specific importin α isoform or isoforms without any affinity for the others, although some NLSs have been shown to have similar affinity for all importin αs (Kohler, 1997). These results suggest that the function of these importins can be controlled in a tissue-specific manner and that distinct substrate specificities exist for importin α. The identification and subsequent naming of importin α isoforms has been complicated due to multiple naming schemes using different numbers for the same importin α and different protein names (Table 1).

Table 1- Importin α Isoforms

Protein	**Importin alpha 1**	**Importin alpha 3**	**Importin alpha 4**	**Importin alpha 5**	**Importin alpha 6**	**Importin alpha 7**
Alternate Protein Names	Karyopherin subunit alpha-2	Karyopherin subunit alpha-4	Karyopherin subunit alpha-3	Karyopherin subunit alpha-1	Karyopherin subunit alpha-5	Karyopherin subunit alpha-6
	SRP1-alpha	Importin alpha Q1	Importin alpha Q2	SRP1-beta		
	RAG cohort protein 1		SRP1-gamma	RAG cohort protein 2		
				Nucleoprotein interactor 1		
Gene Names	KPNA2	KPNA4	KPNA3	KPNA1	KPNA5	KPNA6
	RCH1	QIP1	QIP2	RCH2		IPOA7
	SRP1					
Organism	Homo sapiens	Homo sapiens	Homo sapiens	Homo sapiens	Homo sapiens	Homo sapiens
Sequence Length	529aa	521aa	521aa	538aa	539aa	536aa

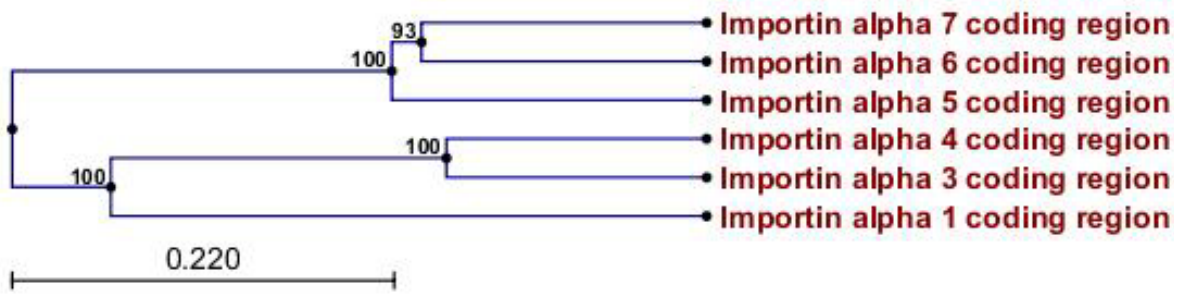

Figure 5-Importin α Alignment Tree calculated on CLC DNA Workbench

Protein-protein interactions

Protein-protein interactions play important roles for almost all biological functions such as transcription, signal transduction pathways, metabolism, development, and other enzyme-mediated reactions. There are many methods available that can be used to detect protein-protein interactions, each with its own strengths and weaknesses. These include affinity blotting, immunoprecipitation, affinity chromatography, and two-hybrid screening to name a few common techniques (Phizicky and Fields, 1995). The choice of which method to be used depends on a variety of factors such as the ability to purify a protein, previous knowledge on the protein-protein interaction, and availability of specific antibodies.

Mammalian Two-Hybrid

Two-hybrid screening was a method first introduced in order to accelerate the rate of progress in identification of novel protein-protein interactions. Two-hybrid screening was first developed in yeast and took advantage of the fact that transcription factors have a modular structure containing both an activation domain and a DNA binding domain. These modular transcription factors possess separate functional domains that can be expressed individually from recombinant vectors. Transcription will be activated when these two proteins become closely associated with one another. This allows one to fuse a protein of interest (X) with the DBD and another protein of interest (Y) with the activation domain in order to determine if proteins X and Y interact *in vivo*. When proteins X and Y interact the activation domain and DBD become very closely associated

and the two functional domains together enable activation of gene expression when placed in the proximity of a target promoter.

Two-hybrid screening has also been adapted for use in mammalian cells in order to create an environment that permits certain post-translational modifications to occur. One mammalian two hybrid system, the CheckMate system by Promega, uses the DNA binding domain of the GAL4 protein and the activation domain of herpes simplex virus type 1 VP16 protein. These proteins provide functional transcriptional activation from RNA polymerase II basal promoters with upstream GAL4 binding sites. The GAL4-VP16 hybrid protein has been shown to activate transcription with an unusually high efficiency in mammalian cells (Sadowski, 1998). The GAL4 fusion protein gene is located within the pBIND vector and the protein of interest is cloned in frame with GAL4. The pBIND vector also contains a *Renilla* luciferase gene in order to monitor the transfection efficiency of the pBIND vector. The VP16 fusion protein gene is located within the pACT vector and the second protein of interest is cloned in frame with VP16. The pACT vector also contains a neomycin gene in order to select for cells that were successfully transfected with the pACT vector. The GAL4 binding site is within the PG5luc vector and is located upstream of a firefly luciferase gene that is used as a reporter gene. The level of luciferase can be assayed in order to determine the binding affinity of the two proteins in question, X and Y (Schenborn and deBerg, 1998). Mammalian two hybrid screening creates a system to study protein interactions that closely resembles a cellular environment that mimics the native protein environment. It differs from the yeast two hybrid as certain post-translational modifications such as glycosylation, phosphorylation, and acylation will occur in a mammalian cell, but will

not in a yeast cell (Buckholz and Gleeson, 1991). Many protein interactions depend on these post-translational modifications and will not occur without them. Also sometimes other cellular proteins are required in order for two proteins to interact so an environment that is similar to the native protein environment is more likely to have these factors than a yeast environment.

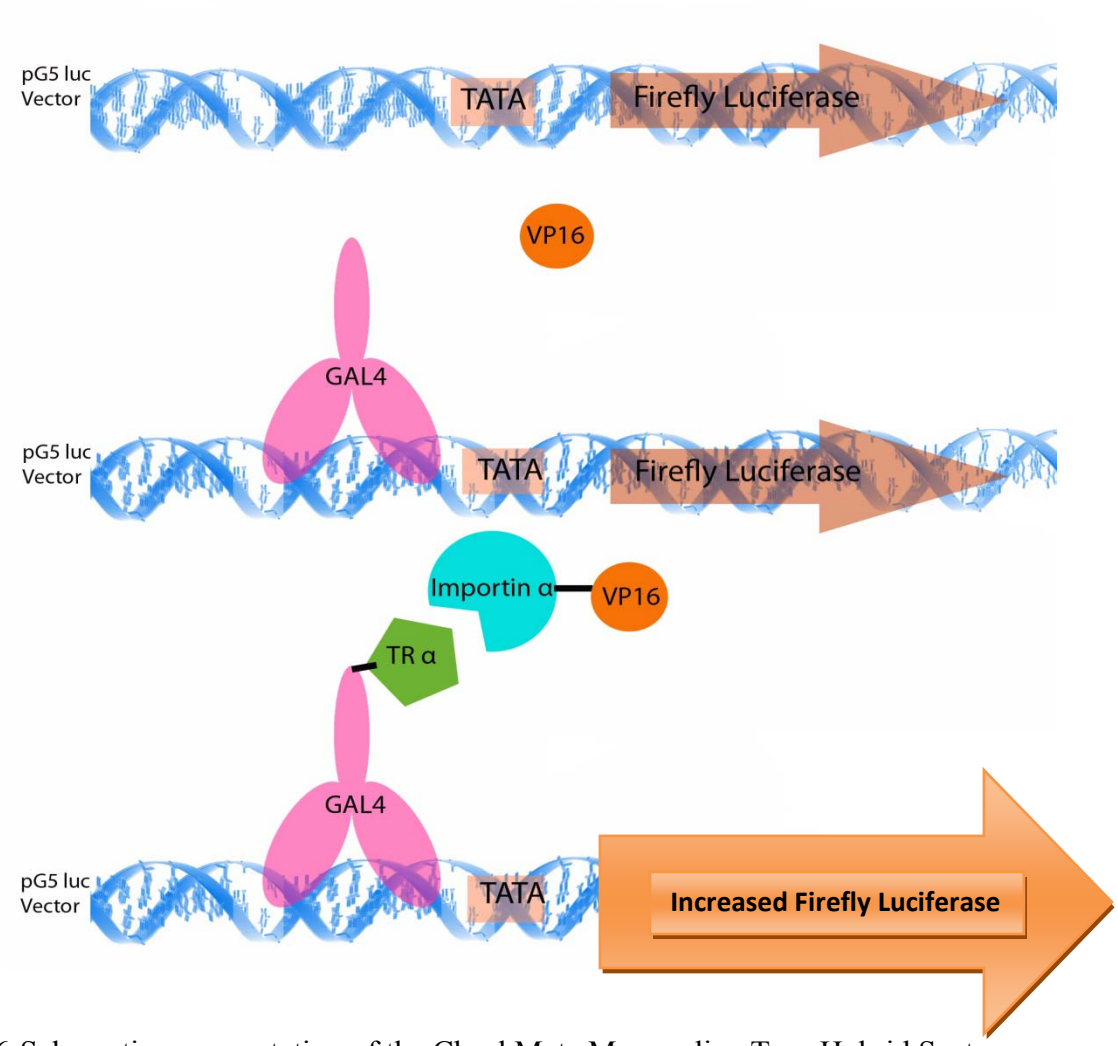

Figure 6-Schematic representation of the CheckMate Mammalian Two-Hybrid System

The pG5luc vector contains a GAL4 binding site upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. The upper diagram shows the vector by itself without the pACT and pBIND vector. The middle diagram shows the pACT and pBIND vectors transfected along with the pG5luc vector. The lower diagram shows the proteins of interest fused into their respective vector and this interaction will result in an increase in firefly luciferase expression.

Specific Aims of Research

The overall aim of this thesis research was to investigate the nuclear import pathway followed by thyroid hormone receptor alpha by studying binding affinities of individual TR alpha domains for the different importin alpha isoforms. A method to study this interaction needed to be developed and optimized for use. This leads to a series of specific questions that were addressed:

1) Can the expression and purification of TR and various transport factors be optimized in order to produce enough protein for *in vitro* binding studies?
2) Can the mammalian two-hybrid system be used to study the interactions between a transcription factor and importin, more specifically TRα and importin α?

Methods

Plasmids and Subcloning

The plasmids pQE-30 Xa-GFP TR AB, pQE-30 Xa-GFP TR DBD, pQE-30 Xa-GFP TR Hinge, and pQE-30 Xa-GFP TR LBD were constructed by subcloning the PCR products GFP TR AB, GFP TR DBD, GFP TR Hinge, and GFP TR LBD (from previous GFP-GST-GFP TR alpha domain vectors made by M.S. Mavinakere) into the *E coli* protein expression plasmid pQE-30 Xa (Qiagen, Germantown, MD) using BamHI and SphI restriction enzymes (NEB, Ipswich, MA). Pfu Turbo (Agilent, Santa Clara, CA) was used to PCR the GFP-tagged domains and BamHI and SphI ends were added to the primers. After restriction enzyme digests, the DNA was purified using QIAquick PCR Purification Kit (Qiagen, Germantown, MD) per manufacturer's protocol and

subsequently electrophoresed on a 0.7% or 10.5% agarose gel depending on the size, and the precise bands were excised and purified using QIAquick Gel Extraction Kit (Qiagen, Germantown, MD). Following gel purification the digested DNA fragments were ligated together using T4 DNA Ligase (NEB, Ipswich, MA) per manufacturer's protocol and subsequently transformed into NEB's 5-alpha Competent *E coli.* (High Efficiency). Colonies were minipreped using QIAprep Spin Miniprep Kit (Qiagen, Germantown,MD) per manufacturer's protocol and screened by assessing the size on a 0.7% agarose gel.

Plasmids pACT, pBIND, pG5luc, pBIND-ID, and pACT-MyoD were all commercially prepared and purchased in the Checkmate Mammalian Two-Hybrid Kit by Promega. Importin alpha isoforms 1,3,4,5,6,7 lacking the IBB domain were subcloned in frame with the VP16 gene located in the pACT vector and TR alpha domains were subcloned in frame with the GAL4 gene by ligating the PCR products (from vectors made by Hunziker and Mavinakere, respectively) into pACT and pBIND, respectively, using EcoRV and NotI restriction enzyme sites (NEB). The PCR products were digested with NotI and purified as described above. The purified, digested importin alpha and TR alpha domain PCR products were phosphorylated using T4 Polynucleotide Kinase (NEB) per manufacturer's protocol. pACT and pBIND vector were digested with EcoRV and NotI to create blunt and sticky ends for ligation of importin alpha and TR alpha domain PCR products respectively. The digested vectors were purified as described above. The digested vectors were then dephosphorylated using Antartic phosphatase (NEB) per manufacturer's protocol in order to reduce vector religation. The vectors and PCR fragments were ligated using the same method as described above. The subsequent colonies were miniprepped and sequenced by the DNA Analysis Facility on Science Hill

at Yale University in order to determine if the PCR fragment was cloned in frame. Colony screening was performed by using a fast and impure mini prep protocol in brief. 2 ml of overnight culture was microcentrifuged at 13,000 rpm for two minutes and the supernatant was discarded. 300 µl of TENS buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, .1 N NaOH, 0.5% SDS in 100 ml ddH_20) was added to the bacterial pellet and set on ice for 10 minutes. 150 µl of 3.0 M Sodium Acetate, pH 5.2 was added to the mixture and vortexed. Next the tube was microcentrifuged for 6 minutes at 13,000 rpm. The supernatant was then transferred to a new tube containing 0.9 ml of 100% EtOH precooled to -20°C and then microcentrifuged for 2 minutes. The supernatant was discarded and the DNA pellet was rinsed with 70% EtOH and microcentrifgued once again for 2 min. These mini preps were run on a 0.7% agarose gel to determine which colonies had the inserted fragment.

The plasmid pGEX-CRTwt was a gift from B. Paschal (University of Virginia) and encodes full length calreticulin subcloned into the pGEX-KG vector for overexpression in *E coli* (Holaska et al., 2001).

The plasmids pGEX-6P-1-Importin alpha 1,3,4,5,6, and 7 were a gift from W. Hunziker (Institute of Molecular and Cell Biology, Singapore) and they encode full length importin alpha subcloned into a pGEX-6P-1 vector for overexpression in *E coli* (Mahalakshmi et al., 2007).

All plasmids were propagated in *E.coli*-DH5α using either LB broth (1% peptone, 0.5% yeast extract, and 1% NaCL) or Super Broth (3.2% peptone, 2% yeast extract, and 0.5% NaCl) and purified using a Qiagen Plasmid Midi Kit or Qiagen Miniprep Kit

(Qiagen Inc., Valencia CA). DNA purity and concentration were measured using a NanoDrop® ND-1000 full-spectrum UV/Vis Spectrophotometer.

Protein Overexpression in *E coli*

Plasmids coding for the protein of interest were transformed into competent *E coli (*BL21 DE-RIL) (Stratagene, LaJolla, CA), per the manufacturer's protocol and grown at 37°C. 3ml of media was inoculated with a single colony and grown for ~8 hours with shaking and a new 25ml culture was inoculated with 250 ul of the starter culture. The 25ml culture was grown overnight to saturation with shaking and subsequently diluted 1:10 in fresh media. This 250 ml culture was then grown for varying temperatures (18°, 20°, 22°, 25°, 30°, and 37°C) for 1 to 4 hours with shaking. This 250 ml culture was then induced by adding IPTG to a final concentration which was also varied from 0.1 to 1.0 mM. Cells were then centrifuged for 15 min at 5000 rpm at 4°C. The supernatant was then discarded and the bacterial pellets were then stored at -80°C until purification. Various media were used in order to increase protein expression including LB Lennox Broth (1% peptone, 0.5% yeast extract, and 0.5% NaCl), Terrific Broth (1.2% peptone, 2.4% yeast extract, 72 mM K_2HPO_4, 17 mM KH_2PO_4, and .4 % glycerol), 2x YT Broth (1.6% peptone, 1% yeast, and 0.5% NaCl), and SOC medium (2% peptone, 0.5% yeast extract, 10 mM NaCl, 20.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose). Also varying amount of $ZnCl_2$ and glycerol were added prior to induction in order to promote protein expression.

GFP-tagged Protein Quantification

The emission of GFP-tagged proteins was measured using a Perkin-Elmer LS 55 Luminescence Spectrometer. Crude protein extracts were measured for eGFP activity in order to screen for fusion protein expression. The crude protein extraction was excited at 475nm and the emission was scanned from 480 to 600nm.

GST-tagged Protein Purification

Bacterial pellets were resuspended in 10 ml B-PER® Bacterial Protein Extraction Reagent (Pierce, Rockford, IL), 1 ml 5.0 mg/ml lysozyme (Fisher) 10mM Tris, pH 8.0, and one Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, IN). Resuspended pellets were incubated on ice for 30 min. The pellets were subsequently sonicated 5X at a setting of "6" for 5 to 10 seconds each time (Sonic Dismembrator Model 100; Fisher) on ice to fully lyse the bacteria. The lysed mixture was then centrifuged at 13,000 rpm for 15 min at 4^{o}C, and the supernatant was applied to 200µl of 50% MagneGST Resin (Promega, Madison, WI). Samples were incubated for 60 min at 4^{o}C with gentle rotation and then placed in the magnetic stand to allow MagneGST particles to be captured by the magnet. The supernatant was carefully removed and the resin pellet was washed 3 times with 10 ml ice cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and then transferred to a 10.5 microcentifuge tube and washed an additional 3 times with 600µl ice cold PBS. 100µl Glutathione Elution Buffer (10mM glutathione) was added to the tube, incubated at room temperature for 2-4 min with agitation, and then placed on the magnet stand to collect MagneGST Resin. The elution step was repeated 3X. The eluted fractions were

pooled and dialyzed (Slide-A-Lyzer® Mini Dialysis Units, 7000MWCO, Pierce) against PBS overnight at 4°C. Concentrated protein samples were analyzed by SDS-PAGE using 8% or 12% gels depending on protein size. Protein concentration was estimated using a NanoDrop® ND-1000 full-spectrum UV/Vis Spectrophotometer. Samples were stored at -80°C.

His-tagged Protein Purification

Bacterial pellets were resuspended in 10 ml B-PER® Bacterial Protein Extraction Reagent (Pierce), 1 ml 5.0 mg/ml lysozyme (Fisher), 10mM Tris, pH 8.0, and one Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, IN). The suspension was transferred to a glass Corex tube and subsequently sonicated 5X at a setting of "6" for 5 to 10 seconds each time (Sonic Dismembrator Model 100; Fisher) on ice to fully lyse the bacteria. The lysed mixture was then centrifuged at 13,000 X g for 15 min at 4°C. The supernatant containing soluble proteins was transferred to a 15 ml Falcon tube containing 200 µl of pre-equilibrated 50% Talon resin (Clontech/BD Biosciences). Samples and Talon resin were incubated for 60 min at 4°C with gentle rotation, then centrifuged for 5 min at 500 X g at 4°C to pellet the resin. The resin pellet was washed 3 times with 10 ml ice cold 1X Equilibration/Wash buffer (pH 7.0) (50 mM sodium phosphate; 300 mM NaCl) and then transferred to a Microfilter Spin Column (Pierce) and washed an additional two times with 600µl 1X Equilibration/Wash buffer. 100µl 1X Imidazole Elution buffer (50 mM sodium phosphate, pH 7.0; 300 mM NaCl;150 mM imidazole) was added to the column, incubated at room temperature for 2-4 min with agitation, then centrifuged at 700 X g for 30 sec at 4°C to collect eluted protein. The elution step was repeated 3X. The eluted

fractions were pooled and dialyzed (Slide-A-Lyzer® Mini Dialysis Units, 7000MWCO, Pierce) against D-PBS overnight at 4°C. Protein samples were then concentrated using Micron Ultracel YM-30 Centrifugal Filter Devices (Millipore, Bedford, MA). Concentrated protein samples were analyzed by SDS-PAGE. Protein concentration was estimated using a NanoDrop® ND-1000 full-spectrum UV/Vis Spectrophotometer. Samples were stored at -80°C.

SDS-PAGE

Protein samples were visually analyzed using SDS-PAGE. A BioRad Mini Protean II gel apparatus (Hercules, CA) was set up according to the manufacturer's specifications and acrylamide (30% Acrylamide/Bis solution, 29:1, 3.3% C) gel percentages of either 8% or 12% were used depending on protein size.

Protein samples of varying concentrations were added to 1X SDS-PAGE Sample Buffer (125mM Tris, pH 6.8; 1% SDS; 5% glycerol; 0.005% bromophenol blue; 20mM DTT), and boiled for 5 min to denature the proteins.

All gels were loaded with one lane of Kaleidoscope Protein Standards (5µl) (BioRad). Samples were electrophoresed in 1X SDS-PAGE Running Buffer (192mM glycine; 25mM Tris; 3mM SDS, pH 8.2) at 150 V until the size standard band closest to the protein sample size was midway through the separating phase of the gel.

After electrophoresis, the gel was washed 3X for five min with sterile deionized water. The gel was then immersed in Simply Blue Safe Stain (Invitrogen) for one hour followed by immersion in 100ml deionized water for destaining. Images were captured

digitally using a BioRad Gel Doc XR documentation system with Quantity One analysis software (v4.6.1).

Cell Culture

HeLa cells (ATCC CCL-2) and NIH3T3 cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and D-MEM supplemented with 10% calf serum respectively (Invitrogen, Carlsbad, CA) containing penicillin (10,000U/ml) and streptomycin (100 μg/ml), at 37ºC under 5% CO_2 and 98% humidity. Cells were grown to 70-90% confluency.

Transient transfection

Cells were prepared for transfection when they were >70-80% confluent. First, the media was removed and the cells were rinsed with 10 ml Dulbecco's modified Phosphate Buffered Saline (D-PBS: 0.10 g KCl, 0.10 g KH2PO4, 4.00 g NaCl, and 1.08 g 16 Na2HPO4·7H2O in 500 ml ddH2O). The D-PBS was aspirated and 2 ml 0.25% or .1% depending on cell type trypsin (protease) was added to break down the extracellular matrix and detach the cells from the bottom of the flask. For HeLa and NIH3T3, the trypsin was withdrawn after 2 minutes. After 2 minutes of trypsin exposure the flask was placed in the 37°C incubator for an additional 2-4 minutes. Once the cells had fully detached from the bottom of the flask, the flask was removed from the incubator. HeLa and NIH3T3 cells were diluted in 8 ml of media.

A hemacytometer was used to determine the cell number. Cells were seeded at a optimized number of 3 x 10^5 cells per well into 6-well plates (Corning). Twenty-four

hours after seeding, cells were transfected with 2 μg plasmid DNA and 4 μl LipofectAmine 2000 in Opti-MEM I Reduced Serum Medium (Invitrogen).

Mammalian Two-Hybrid Screening

Either HeLa or NIH3T3 cells were seeded and transfected as above with the following three vectors: pG5luc, pACT, and pBIND with varying inserts (TR and importin α) in pACT and pBIND vectors. After ~12-16 hours the cells were give fresh media treated with neomycin at 6mg/ml and allowed to grow overnight and were again treated with neomycin the next morning. The cells were then assayed for both firefly and *Renilla* luciferase 48 hours after transfection.

Dual-Luciferase Assay

Luciferase activity was measured using the Dual-Luciferase Assay System (Promega) following the manufacturer's protocol and a Femtomaster FB 12 luminometer (Zylux Corp). Briefly, growth media was removed and the cells washed with 2 ml of ice-cold D-PBS. After complete removal of D-PBS, 500 μl of passive lysis buffer was added and the plate incubated at room temperature for 15 min with shaking. After incubation, firefly luciferase activity was measured by adding 20 μl of cell lysate to 100 ul of luciferase assay reagent (LARII) and immediately reading the flash using the Femtomaster luminometer. Next 100 μl of Stop and Glo reagent was added to the mixture and the flash was measured for *Renilla* Luciferase activity.

Results

TRα has been shown to rapidly shuttle between the cytoplasm and the nucleus and with a size of 46kDa, TRα is above the upper theoretical limit for passive diffusion through the NPC. In order to show that a NLS is functional *in vivo* one needs to show that the sequence is necessary for import. This is generally done by mutagenesis experiments where basic amino acids are mutated within the putative NLS. This research has been done in our lab for TRα and is soon to be published. It is also necessary to show that the protein of interest directly interacts with its putative import receptor and that this interaction is mediated by the identified sequence. One of the best ways to show this is by performing direct *in vitro* binding experiments with purified proteins. One of the most commonly used methods for obtaining large quantities of recombinant proteins is by overexpression in bacteria, generally *E coli.* Proteins of interest can be cloned into bacterial expression vectors with affinity tags that enable quick and easy purification. The original objective of this research was to produce both His-tagged TR alpha and GST-tagged importin alpha isoforms for use in *in vitro* binding pull down assays in order to determine the importin alpha isoforms that are involved in the nuclear import of TR alpha. Previous research has shown that expression of TR alpha in bacteria is difficult and generally yields low amounts of functional protein (Roggero, 2008).

Optimization of Protein Expression

Thus, the first task was to attempt to optimize conditions in order to increase the expression of TRα. Even after varying temperatures, growth media, IPTG concentrations, and induction times protein expression was still extremely low (<.05 μg/μl) and did not

produce enough protein for *in vitro* binding studies. It is known that purification and overexpression of nuclear receptors is generally quite challenging. Also others have reported that mammalian TRs have proved very difficult to produce in *E coli* (Diallo et al., 2005 ; Roggero, 2008). Studies have shown that isolated protein domains can be used in place of the full length protein and that individual domain constructs typically have increased expression in bacteria when compared to full length proteins (Powers, 2009; Freedman and Yamamoto, 2004). In order to increase the size of the individual TR alpha domains they were tagged with GFP along with a His affinity tag for purification reasons. The GFP tag provides a way to monitor both protein expression and protein folding. Figure 7 shows how the emission at 510nm is able to detect the GFP fusion protein even though a very small amount was recovered after purification. It is possible that the GFP fusion proteins formed inclusion bodies when overexpressed in *E. coli* and were insoluble requiring denaturing conditions in order to purify them. This sort of purification scheme could yield a greater amount of protein but the protein would not be functional and would be useless for *in vitro* binding experiments. It is also possible that the His affinity tag was in a conformation that hindered the purification process as well. Either way, conditions needed to be optimized in order to purify a sufficient amount of functional TRα protein for binding studies.

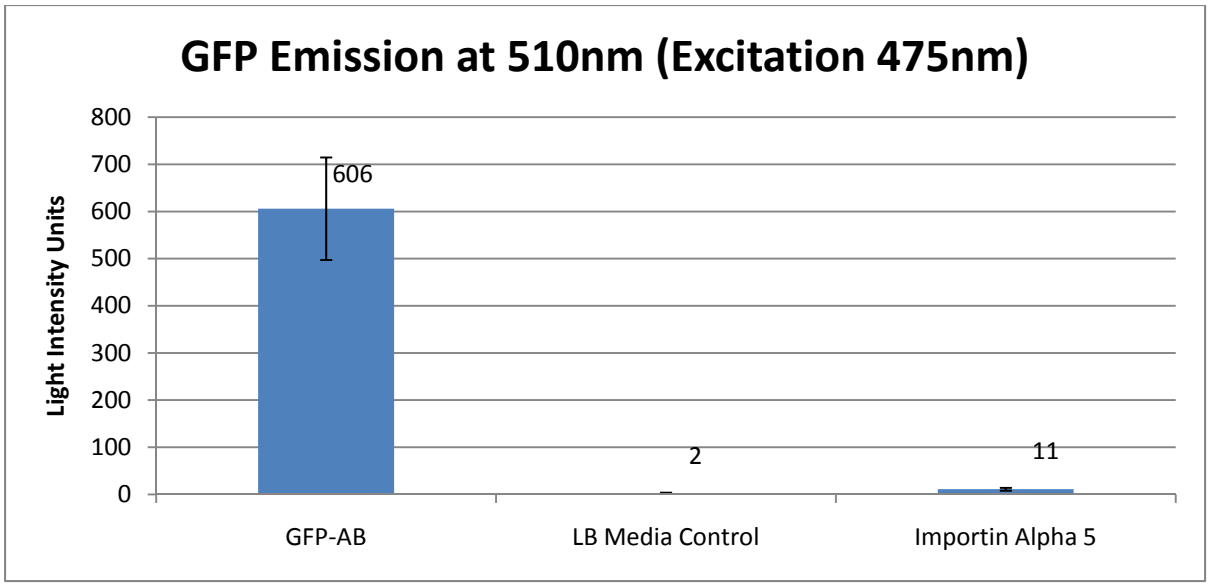

Figure 7- GFP Emission at 510nm

Crude protein extracts and a LB media control, along the x-axis, were excited at 475nm and the emission at 510nm was recorded. Results are expressed as the mean relative light units per sample ± the standard deviation of 6 replicates along the y-axis.

Various conditions were used in order to increase the expression of His tagged GFP TR domains. The first condition that was varied was induction temperature. Although the optimal temperature for *E coli.* growth is 37°C (Fotadar et al., 2005), proper protein folding is often favored under low temperature growth (Baneyx, 1999). Temperatures as low as 18°C were tested for protein expression and it was clear that the lower induction temperatures decreased the growth of the bacteria substantially. Due to the decrease in growth, lower induction temperature cultures were allowed to grow for an increased amount of time when compared to the normal induction times. No substantial differences were seen in the amount of recoverable His-tagged GFP fusion proteins, although lower temperature cultures did show an increase in emission at 510nm. When

the growth media was varied, it was clear that Terrific Broth (TB) was the superior media for protein expression. There was a 3 fold increase in the amount of crude protein in TB cultures when compared to LB cultures. Other factors that were varied, including IPTG concentration and induction time, did not have an effect on the amount of recoverable His-tagged GFP fusion proteins either.

Mammalian Two-Hybrid Optimization

Since purification of both TRα and importin α has proven difficult a new method was needed to study the protein-protein interactions that mediate nuclear import of TRα. The expression of these recombinant proteins has been shown to work well in various mammalian cell lines so an *in vivo* method utilizing mammalian cell lines would be ideal (Powers, 2009). Two-hybrid screening, originally done in yeast, has been shown to work well in various mammalian cell lines and has many pros when done in a mammalian environment.

The CheckMate mammalian two-hybrid system can be used to test for putative protein interactions. The first goal of this research was to optimize the system using control vectors provided with the kit. The two proteins, MyoD and Id, are members of the helix-loop-helix family of nuclear proteins and have been shown to interact *in vitro*. MyoD is a regulator of myogenic differentiation that is expressed in skeletal muscle cells and Id-1 acts as a negative regulator of myogenic differentiation (Benezra et al., 1990). Id-1 is a helix-loop-helix protein that has been shown to be involved in development of cervical, pancreatic, and prostate cancer (Schindl, 2001; Ouyang, 2002). The vectors encoding the fusion proteins GAL4-ID (amino acids 29-148) and VP16-MyoD (amino

acids 1-318) along with the pG5luc vector were all transfected in HeLa cells and ~48 hours later were assayed for firefly and *Renilla* luciferase. As shown in Figure 8, cells transfected with both pBIND-ID and pACT-MyoD control vectors expressed approximately 400 times more firefly luciferase activity than cells transfected with pACT and pBIND Vector combinations. In addition, the level of firefly luciferase activity was very low in cells transfected with vector combinations that did not reconstitute transcriptionally functional GAL4:VP16: pBIND-ID and pACT, pACT-MyoD and pBIND. *Renilla* luciferase expression was used to normalize for transfection efficiency in these experiments in order to standardize the results. These control vectors in combination with pG5luc provide excellent positive control vectors to verify functionality of the mammalian two-hybrid system.

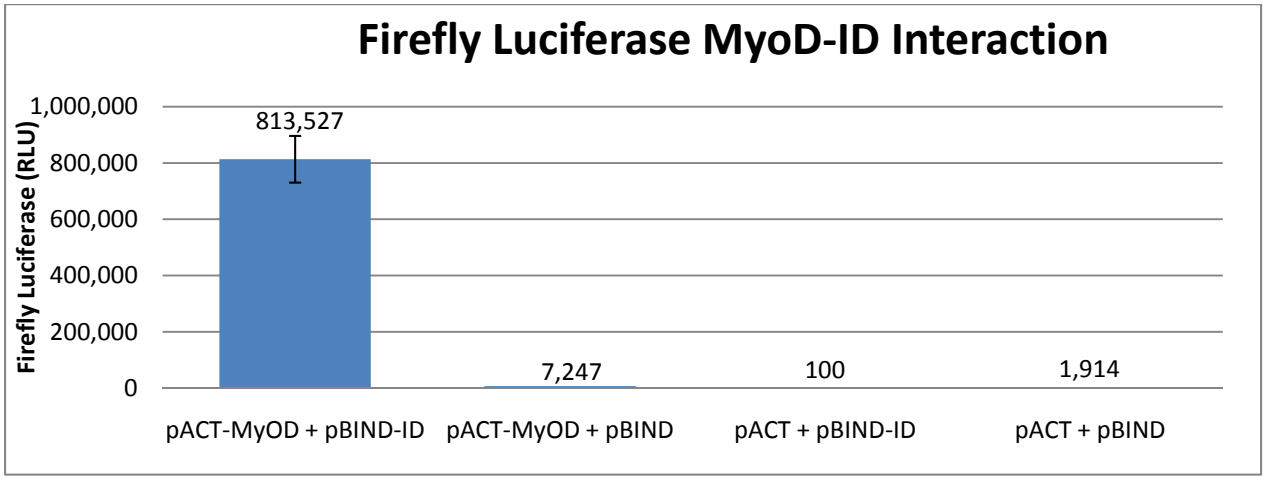

Figure 8- Firefly Luciferase Activity MyoD-ID Interaction

HeLa cells were transfected with the firefly luciferase plasmid pG5*luc* and two control plasmid vectors (pACT vector and pBIND vector) or the corresponding pBIND-ID and pACT-MyoD vectors as described in the Methods. The specific vector combination is shown along the x-axis. The cells were then passively lysed and assayed for luciferase activity. Results are represented as the mean relative light units per sample ± the standard deviation of 6 replicates along the y-axis.

After optimization of the two-hybrid system, subcloned pACT-importin alpha constructs were screened against pBIND-ID to test for interaction between the two. The Id-1, which is a negative regulator of basic helix-loop-helix transcription factors, has been shown to shuttle between the nucleus and cytoplasm. The size of the protein ~18kDa suggests that Id-1 shuttles between the nucleus and cytoplasm simply by passive diffusion; however, Id-1 has been shown to contain both a NLS and NES by localization studies performed with GFP-GFP tagged Id-1. This 2x GFP tag increases the size of the protein in order to stop the passive diffusion of Id-1 (Nishlyama et al., 2007; Kurooka and Yokota, 2005). The NLS located in Id-1 is a novel NLS and does not resemble the cNLS which suggests that Id-1 is not imported by the importin alpha/importin beta complex; however, nothing is currently known about its import pathway. Figure 9 shows the data collected for the interaction between Id-1 and various importin alphas. The firefly luciferase activity was standardized against the luciferase activity of pBIND + pACT. None of the importin alpha isoforms demonstrated a substantial interaction with Id-1, although importin alpha 6 did show a ~40 time increase in luciferase activity when compared to the blank vectors containing GAL4 and VP16 with no inserts. One thing to keep in mind was that the standard deviation was very high, which suggests that this might be a false positive interaction and that more replicates need to be performed before the interaction can be confirmed.

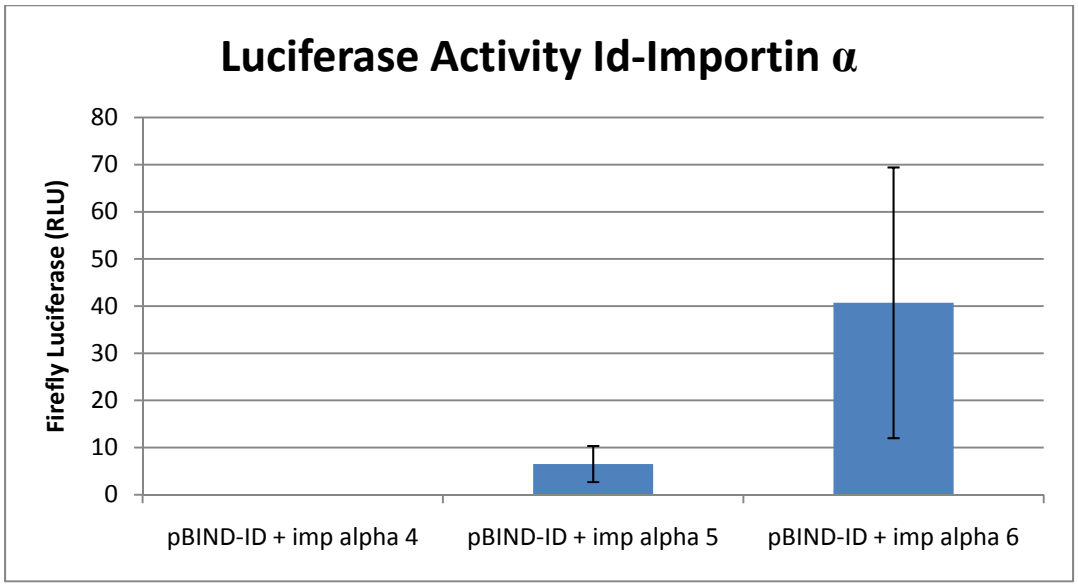

Figure 9- Luciferase Activity Id-Importin α

HeLa cells were transfected with the firefly luciferase plasmid pG5*luc* and pBIND-ID vector along with corresponding pACT-importin alpha vectors as described in the Methods. The specific vector combination is shown along the x-axis. The cells were then passively lysed and assayed for luciferase activity. Results are represented as the mean relative light units per sample ± the standard deviation of 3 replicates along the y-axis.

Some screening of the interaction between TR alpha domains and importin alphas was done although the pBIND-TR alpha domain constructs were never confirmed, due to time constraints, and sequence data was inconclusive on whether or not the inserts were present. The TR alpha domains proved difficult to clone and confirm as most are ~200bp; the insert could not be resolved on an agarose gel as the vector is ~6300 bp. An agarose gel does not provide the resolution needed in order to determine positive clones. This made rapid screening of multiple colonies very difficult with little to no assurance that

the insert was present, since more time consuming PCR or sequencing would need to be carried out.

To summarize, the mammalian two-hybrid system was optimized for use in both HeLa and NIH3T3 cells. This system, in principle, will allow the screening of TRα against various importins in order to determine which importins have a high affinity for TRα, which in turn suggests the molecular pathway that TRα follows to enter the nucleus. More subcloning and colony screening is needed as the TRα domains were unable to be cloned into the pBIND vector, but importin α constructs were subcloned effectively and were confirmed by sequencing.

Discussion and Future Direction

Nuclear import is essential for TR and other transcription factors in order to have an effect on gene regulation. To further understand the molecular mechanisms behind the nuclear transport of TR, binding studies need to be done to determine the importin that mediates nuclear transport. *In vitro* binding studies have proven difficult to do as both TRα and importin α isoforms are difficult to express in *E coli.* In this research, optimization of induction conditions in *E coli* in order to increase expression of functional fusion proteins has proven futile as both TRα and importin α have not been able to be successfully purified from *E coli.* Optimization of GFP tagged TRα domains has proved somewhat successful although purification of these fusion proteins has still proven difficult. These TRα domain fusion proteins were cloned into a bacterial expression vector that encodes a His tag for purification. It is possible that the purification process is not working efficiently due to a number of factors such as the

fusion protein is insoluble, the His tag is being blocked or covered in the 3-D protein structure, or that the proteins are toxic to the bacteria. Both TRα and importin α are proteins that are not found in bacteria suggesting that the proteins could very well be toxic and that the bacteria are degrading the protein (Baneyx, 1999).

There are a few possible future directions that should be followed. First, expression in another organism either yeast or a mammalian cell line would most likely increase expression and the amount of functional protein that can be purified. Another route would be to subclone the importin α and TR domain genes into a different bacterial expression vector that contains a different affinity tag. There are vectors available that contain other larger affinity tags and have been shown to increase expression by increasing solubility of the fusion protein. Tags such as MBP, NusA, and SET have been shown to enhance solubility, whereas both GST and His tags have been shown to have no effect on solubility. Also, other affinity tags offer a different means of purification that might prove more successful than the purification of His tagged proteins (Waugh, 2005).

Since purification of both TRα and importin α has proven difficult a new method was needed to study the protein-protein interactions that mediate nuclear import of TRα. The expression of these recombinant proteins has been shown to work well in various mammalian cell lines so an *in vivo* method utilizing mammalian cell lines would be ideal. Two-hybrid screening, originally done in yeast, has been shown to work well in various mammalian cell lines and has many pros when done in a mammalian environment. When studying protein-protein interactions it is best to study the interaction in the closest environment as possible. The mammalian two-hybrid allows for the study of protein-

protein interactions in an environment that is very similar to the ones encountered by the proteins of interest (Cho et al., 2009; Xie et al., 2009).

The two-hybrid screening first needed to be optimized for the cell line and transfection procedure in our lab in order to be used to study the interactions of interest. The two-hybrid kit used in this research came with two control proteins that can be used to optimize conditions prior to the screening of the proteins of interest. Id-1 and MyoD have been shown to interact *in vivo* and have shown a very strong affinity for one another, thus providing an excellent control for the system. The system was optimized for use in both HeLa and NIH3T3 cells using transfection conditions as described in the Methods. Id-1 and MyoD showed a very strong interaction, over 400 fold increase in luciferase activity when compared to the negative controls containing only the GAL4 and VP16 proteins without inserted proteins. The interaction between Id-1 and importin α 4, 5 and 6 was also tested to see if any interaction could be detected in order to further validate the system. The interactions were for the most part very low and did not show a strong interaction between Id-1 and importin α 4 and 5. The interaction between Id-1 and importin α6 did show a slight increase in luciferase activity when compared to the negative controls. The number of replicates was low (n=3) and the standard deviation was relatively high; thus, more replicates are needed to determine if the interaction is valid.

Also of note was the transfection efficiency which was monitored by the level of *Renilla* luciferase activity in the samples. The transfection efficiency showed a large variation at times and was especially low in most wells that contained pBIND plasmid that was purified in the lab by midipreps as opposed to the plasmid that was purified by Promega in the kit. It is possible that the system requires exceptionally pure plasmid

DNA which was not achieved simply by a midiprep or that the DNA concentrations were not accurate, or could be that the cells were not in the optimal state for transfection. Also using plasmid DNA that was purified from a miniprep also showed very low transfection efficiency. Future direction should be given to purification of plasmid DNA in order to increase transfection efficiency and to also use more careful cell culture techniques. This plasmid purification will also help continuation of the subcloning of TR α domains into the pBIND vector as some samples sent to be sequenced showed a very low quality making them insufficient in determining if the insert was present.

The mammalian two-hybrid is a very strong and powerful means of accessing protein-protein interactions and will provide an excellent means of studying the nuclear import of TRα by screening various importins including other members of the importin β family (e.g. Imp 5, Imp 7, and Imp 13) looking for any interactions. This mammalian two-hybrid can also be used to study nuclear export of TRα as well. After interactions can be shown, the next step would be to use mutagenesis to mutate the suspected NLS sequence to see if the binding affinity is affected. This sort of mutagenesis has been done in our lab on both NLSs in order to study the localization of these mutants, so these mutants could be cloned into the mammalian two-hybrid vectors in order to study the binding affinity with various importins. These experiments in combination with RNAi knockdown studies currently being done in the lab will be instrumental in determining the import pathway that TRα follows.

Significance and Conclusions

TRα function depends on nuclear localization, and both nuclear import and nuclear export of TR are tightly regulated by interactions with various proteins. Nucleocytoplasmic transport is a critical process that provides the cell with an additional level of gene regulation. A cell is able to respond to various stimuli by regulating the subcellular localization of various transcription factors and thereby can regulate protein expression in a cell specific manner. The study of both nuclear import and export is import to understand many cellular processes and is critical in order to understand the molecular mechanism of many diseases. Both the nuclear import and export of various transcription factors have been targets for small molecule drugs that have been developed as therapies for a number of diseases including cancer (Yashiroda and Yoshida, 2003). Also use of NLS sequences as a gene delivery and oligonucleotide delivery system has been proposed for therapeutic use (Lochmann et al., 2004).

TRα is a shuttling protein that must enter the nucleus to bind to TREs in order to regulate gene expression. It is critical to elucidate the molecular mechanism of TRα nuclear import so that the cellular T3 response can be understood fully. Also it is important to understand the shutting of TRα so that any non-genomic actions that TRα might be involved in can be understood as well. This thesis research, along with future work done to study the nuclear transport of TRα, will help understand the molecular pathways that TRα follows in order to enter and exit the nucleus.

Appendix

GFP-TRα domains Primers BamHI/SphI

All Sense primers are exactly the same

GFP A/B Domain

Sense

5’ CGC**GGATCC**ATGAGTAAAGGAGAAG 3’

AntiSense

5’ ACAT**GCATGC**CAGTTATCTAGACTG 3’

GFP DBD

Sense

5’ CGC**GGATCC**ATGAGTAAAGGAGAAG 3’

AntiSense

5’ ACAT**GCATGC**CAGTTATCTAGATAG 3’

GFP Hinge Domain

Sense

5’ CGC**GGATCC**ATGAGTAAAGGAGAAG 3’

AntiSense

5’ ACAT**GCATGC**CAGTTATCTAGATCG 3’

GFP LBD

Sense

5’ CGC**GGATCC**ATGAGTAAAGGAGAAG 3’

AntiSense

5’ ACAT**GCATGC**TTATCTAGAGACTTC 3’

BamHI and SphI recognition sites are in red and blue respectively

TRα Domains Primers for EcoRV/NotI

A/B

Sense

5’ **A**ATGGAACAGAAGCCAAGCAA 3’

AntiSense

5’ ATAAGAATGCGGCCGCACTGCTCGTCTTTGTC 3’

DBD

Sense

5’**A**TGTGTCGTGTGTGGGGA 3’

AntiSense

5’ ATAAGAATGCGGCCGCTAGAACCAGGTCCAT 3’

Hinge

Sense

5’ **A**GATTCAAAGCGGGTGG 3’

AntiSense

5’ATAAGAATGCGGCCGCTCGCCTCTGTTTCCAAT 3’

LBD

Sense

5’ **A**AAATTCCTGCCGGATGACAT 3’

AntiSense

5’ ATAAGAATGCGGCCGCGACTTCCTGATCCTCAA 3’

Importin Alpha Primers for EcoRV/NotI

Importin Alpha 1

Sense

5’ **A**ACTTCTCCGCTGCAGGAAAA 3’

AntiSense

5’ ATAAGAATGCGGCCGCCTAAAAGTTAAAGGTCCCAGGAGCC 3’

Importin Alpha 3

Sense

5’ **A**GACTCTGATATAGATGGTGA 3’

AntiSense

5’ ATAAGAATGCGGCCGCCTAAAACTGGAACCCTTCTG 3’

Importin Alpha 4

Sense

5’**A**GATTCAGATGTTGATGCTGA 3’

AntiSense

5’ ATAAGAATGCGGCCGCTTGAAGGTTGGCTGTTG 3’

Importin Alpha 5

Sense

5’ **A**GAAGAAGAAGTTATGTCAGATGGAGGC3’

AntiSense

5’ATAAGAATGCGGCCGCTCAAAGCTGGAAACCTTCCATAGGAG 3’

Importin Alpha 6

Sense

5’ **A**TCTATGCTTGAAAGTCC 3’

AntiSense

5’ ATAAGAATGCGGCCGCTTAAAGTTGAAATCCATCCATTGGTGC 3’

Importin Alpha 7

Sense

5’ **A**GCCATGTTCGATAGTCTTCT 3’

AntiSense

5’ ATAAGAATGCGGCCGCTTATAGCTGGAAGCCCTCCA 3’

NotI recognition sites are highlighted in yellow;

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